

Full Length Research Paper

Optimization of embryogenic-callus induction and embryogenesis of *Glycyrrhiza glabra*

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Accepted 21 April, 2010

Glabridin is a major biologically active flavonoid isolated specifically from the root of *Glycyrrhiza glabra*, which has many pharmacological activities. The production of the wild *G. glabra* was sharply decreased due to immoderate and ruinous utilization. *In vitro* regeneration via somatic embryogenesis is important for clonal propagation and genetic transformation. In this paper, factors affecting the embryogenic calli and embryo induction, maintenance and multiplication of *G. glabra* are assessed. The results showed that the explants of hypocotyl give the highest calli formation frequency of 93.3% on Murashige and Skoog (MS) medium containing 2.0 mg/L 6-benzylaminopurine (6-BA) and 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). The maximum efficiency of embryo were obtained on MS medium with 0.5 mg/L 6-BA + 0.5 mg/L kinetin zeatin (KT) + 0.1 mg/L indole-3-butyric acid (IBA); the embryos could develop further on medium with 1000 mg/L malt extract (ME). The occurrence of the embryogenic calli and proglobular embryo were studied by histological section, indicating the single cell origin of the embryogenesis of *G. glabra*. With the protocol reported herein, some green embryo-like cultures were obtained, from which shoots were successfully regenerated in the germinated medium after 10 months of subculture.

Key words: *Glycyrrhiza glabra* L., callus induction, embryogenesis, cell culture, histological section.

INTRODUCTION

Licorice, the root of the leguminous *Glycyrrhiza* plant species (*Glycyrrhiza uralensis* Fisch., *Glycyrrhiza inflata* Bat., *Glycyrrhiza glabra* L.), has been used as food and traditional medicine over 4000 years. Glabridin is a major biologically active flavonoid included specifically in *G. glabra* and has antioxidant activity (Hong et al., 2009), anti-*Helicobacter pylori* activity (Fukai et al., 2002), antifungal activity (Fatima et al., 2009) etc. Unfortunately, the yield of the wild *G. glabra* is severely reduced owing to the immoderate and ruinous utilization and it has been

considered as the fourth protected plant in Mongolia. Therefore, it is urgent to obtain sufficient products to supply the market by planting artificially; but the regeneration rate of the seed of wild *G. glabra* is very low (only 5–10%). Accordingly, new approaches of producing *G. glabra* are necessary to meet the increasing demands.

In vitro regeneration via somatic embryogenesis is important for clonal propagation and is usually an integral part of genetic modification (Varisai et al., 2004; Huo et al., 2005). Although work concerning *in vitro* cell and tissue culture researches have been extensively reported on the other *Glycyrrhiza* plant species such as *G. uralensis* Fisch (Yu et al., 1999; Huo et al., 2005), to our knowledge, there is only few reports on the micropropagation of *G. glabra* through shoot tip and nodal cultures (Thengane et al., 1998; Mousa et al., 2006). So far, information on callus induction and embryogenesis of *G. glabra* is largely unknown.

The present investigation reports a detailed study on the callus induction, embryogenesis of *G. glabra* by investigating the factors affecting the callus induction and

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Abbreviations: BA, 6-Benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; IBA, indole-3-butyric acid; KT, kinetin zeatin; ME, malt extract; NAA, naphthaleneacetic acid; PGR, plant growth regulators; MS, Murashige and Skoog.

These Authors contributed equally to this work.

Table 1. Influence of differential hormone combination on callus induction of *G. glabra*.

Treatment	6-BA (mg/L)	2,4-D (mg/L)	NAA (mg/L)	KT (mg/L)	IBA (mg/L)	Callus induction rate (%)
C1	0.5	0.5	0.5			84 ± 2.31ab
C2	2.0	0.5				93.3 ± 1.16a
C3	1.0	0.5				88.1 ± 1.73a
C4	0.5	0.5				83.3 ± 1.89ab
C5	0.5			0.5	0.1	42.2 ± 1.33c

The basic medium in C1 - C5 is MS medium. Values are the mean number (± S.E.) of callus induction in three replicates. The different letters are significantly different at $P < 0.05$ with Duncan's multiple range tests.

Table 2. Influence of differential hormone combination on somatic embryogenesis of *G. glabra*.

Treatment	6-BA	NAA	2,4-D	KT	IBA	ΔFW (mg)	Embryogenesis
E1	0.5	0.5	0.5			12.0 ± 0.4a	1
E2	0.5	0.2	0.2			10.5 ± 0.6a	2
E3	0.5			0.5	0.1	8.0 ± 0.3ab	3
E4						7.0 ± 0.5b	0

The basic medium in C1- C5 is MS medium. Values are the mean number (± S.E.) of ΔFW in three replicates. The different letters are significantly different at $P < 0.05$ with Duncan's multiple range tests. "0" degree refer to no embryogenesis, only white or yellow white callus; "1" degree refer to that green area is below 30%; "2" degree refer to pea green, but the green area is around 30 to 70%; "3" degree refer to dark green and green area is above 70% .

embryogenesis of *G. glabra*. In addition, the occurrence characteristic of the callus and embryos was also studied via microscopic observation. Our work aims to establish an efficient protocol for inducing somatic embryogenesis from *G. glabra* L. that can be used for genetic engineering in the future.

MATERIALS AND METHODS

Material

The seeds of *G. glabra* were kindly provided by Xinjiang Kun-Shen Corporation.

Asepsis explant preparation

The seeds of *G. glabra* were rinsed in water for 30 min and the fully matured seeds were treated with 98% oil of vitriol for about 30 - 40 min and washed five times using sterile water. The seeds were then surface sterilized for 8 - 10 min by 1% high mercury bichloride, followed by wash with sterile water for three times. Lastly, the seeds were cultured on MS (Murashige and Skoog, 1962) solid medium in the dark at 25°C

Callus induction

The effects of different explants and plant growth regulators (PGR) on callus induction were examined using two independent experiments. Firstly, the effects of different explants such as hypocotyl, cotyledon, young leaf and stem segment on callus induction were evaluated

on C1 medium containing 0.5 mg/L 6-benzylaminopurine (6-BA), 0.5 mg/L 2,4-D, 0.5 mg/L NAA and C2 medium containing 2.0 mg/L 6-BA, 0.5 mg/L 2,4-dichloro-phenoxyacetic acid (2,4-D). Secondly, 5 hormone combinations were designed using different content of 6-BA and 2,4-D (Table 1). All treatments were carried out at least twice with three repetitions (15 explants for each). After 30 days, the formation rate and quality of callus were investigated. The growth of callus was recorded as the percent explants forming callus.

Callus formation rate (%) = Number of callus/incubated explants x 100

Somatic embryogenesis and development

To induce somatic embryos, clear and glandular calli derived from hypocotyl were transferred to MS medium containing different PGR (Table 2). All treatments were carried out at least in duplicate with three repetitions (about 7 g calli were used in each treatment). After 30 days, the callus multiplication index and embryogenesis rate were measured.

The embryogenesis rate was recorded for the percentage of somatic embryos at globular stage. "0" degree refers to no embryogenesis, only white or yellow white callus; "1" degree shows that the green area is below 30%; "2" degree refers to pea green, but the green area is around 30% to 70%; "3" degree refers to dark green and the green area is above 70%.

For promoting full development of the globular embryos, they were transferred to MS medium supplied with 0.5 mg/L 6-BA, 0.5 mg/L kinetin zeatin (KT), 0.1 mg/L indole-3-butyric acid (IBA), added with 500 mg/L, 1000 mg/L or 1500 mg/L of malt extract (ME). After 30 days of transfer, the embryo multiplication index and development were measured. The work was repeated 3 times.

The well-developed dark green globular embryos were cultured

on MS basic medium supplemented with 3.0 mg/L 6-BA, 1.0 mg/L KT, 0.1 mg/L naphthaleneacetic acid (NAA) for shoot induction.

Histological analysis

Histological studies of embryogenic callus, non-embryogenic callus and embryo were carried out by paraffin section and semi-thin section methods. Paraffin sections were prepared according to Hartweck et al. (1988) with some modifications. The samples were fixed for 24 - 48 h at room temperature by FAA fixative solution (formalin: glacial acetic acid: 70% ethanol, 5: 5: 90 by volume), followed by dehydration in a graded ethanol series (30, 50, 70, 85, 95 and 100%) and embedded in paraffin. A 12- μ m thick section was cut from each paraffin block containing the corresponding sample with KD-1508A rotary Microtome and fixed to glass slides. The sections were dried at 40°C for 3 days and deparaffinized with 100% xylene, followed by staining with 0.5% (w/v) fast green and 0.25% (w/v) safranin and examined under a microscope. For preparation of semi-thin sections, the collected samples were first treated as paraffin section as mentioned above, then embedded in Epon812. Samples were sectioned at a 8- μ m thickness by a rotary Microtome stained with 1% Toluidine Blue O (Aldrich, Milwaukee), examined under a microscope (BH-2, Olympus) and photographed with an Olympus DP70 CCD camera.

Statistical analysis

All experiments were arranged in a completely randomized design. Cultures were observed monthly, the percentage of explants forming callus, somatic embryo induction and mean callus weight were analyzed using analysis of variance (ANOVA) and Duncan's multiple range test and significance was determined at $p = 0.05$ level.

RESULTS AND DISCUSSION

Effects of explant type on *G. glabra* L callus induction

Five days after the inoculation of the seeds, the 4 - 5 cm high seedlings were transferred to light; 3 days later, the seedlings (Figure 1A) were used as a source of hypocotyl, cotyledon, young leaf and stem segment. The hypocotyl was cut into segments with 1 cm in length. Explants of hypocotyl, cotyledon, young leaf and stem segment were used to induce callus on C1 and C2 medium. The results showed that callus can be induced from all of the tested explants within 3 weeks of culture, but the morphology of the calli induced from different explant are highly various. Callus from hypocotyl was clear and glandular, particularly at the cutting surface (Figure 1B). White or light brown and friable callus with a shiny surface was induced from cotyledon, while white or light brown, soft callus could be induced from young leaf explants at the cutting ends. The callus from stem segment was extremely hard and dark brown and stopped growth within 16 weeks. Hypocotyl gave rise to the highest callus formation frequency and intensity on induction medium, whereas the lowest frequency was observed in stem segments. The C2 medium has a higher inducing ability of 48.7 - 94.3% than C1 (42.7%~86.3%) (Figure 2).

Effects of PGR on callus formation

The exogenous 2,4-D is the major factor affecting cell dedifferentiation and it was confirmed that the combination of 2,4-D and other suitable PGRs showed a better effect than sole 2,4-D. In our pre-experiment, 0.5 mg.l⁻¹ 2,4-D is better for calli induction from hypocotyl of *G. glabra*. The effects of media with a combination of 2,4-D and different hormone on the callus induction from hypocotyl of *G. glabra* were examined in this research. The results (Table 1) showed that a high concentration of 6-BA combined with 2,4-D led to a better results for calli induction, with the highest callus forming efficiency of 93.3% being noted in MS medium containing 6-BA (2.0 mg/L) and 2,4-D (0.5 mg/L). Within 2 weeks on this medium, the embryogenic calli consisting of dense cytoplasm, small vacuoles and large nuclei with deeply stained nucleus were formed (Figures 1C and 3F). Such calli could be maintained for over 1 year by monthly subculture and 1.5 - 2 fold increases in FW (fresh weight) were obtained for 1 month, otherwise, the callus induction efficiency is lower in the low 6-BA content combined 2,4-D treatment (Table 1), and the induced calli easily turned brown or died when subcultured. Our results showed that high concentration of 6-BA were necessary for the callus induction of *G. glabra* and the supplementation of NAA could not improve the callus formation rate when 6-BA content was low, which is inconsistent with previous reports on *G. uralensis* Fisch (Huo et al., 2005).

Influence of PGRs on somatic embryogenesis of *G. glabra*

Induction of somatic embryogenesis differed with PGR combinations. Although 2, 4-D helped in the maintenance of the calli (Figure 1C), it was unfavorable for the induction and development of the embryos. It was noted that somatic embryos cultured on MS medium containing low concentration of 2,4-D formed callus again, in line with what has been reported for horsegram (*Macrotyloma uniflorum*) (Mohamed et al., 2005). In the current case, the rate of somatic embryogenesis is higher on the medium without 2,4-D (Table 2) and the maximum was obtained in MS medium containing 6-BA (0.5 mg/L), KT (0.5 mg/L) and IBA (0.1 mg/L). On this medium, many green globular somatic embryos were observed on the calli surface 15 days later after the inoculation (Figure 1D) and the embryos were easily separated from the surrounding calli.

The effects of additives on the embryo multiplication and development of embryo of *G. glabra*

In the previous investigation, it was shown that the embryo could not grow further on the same 6-BA (0.5 mg/L) + KT

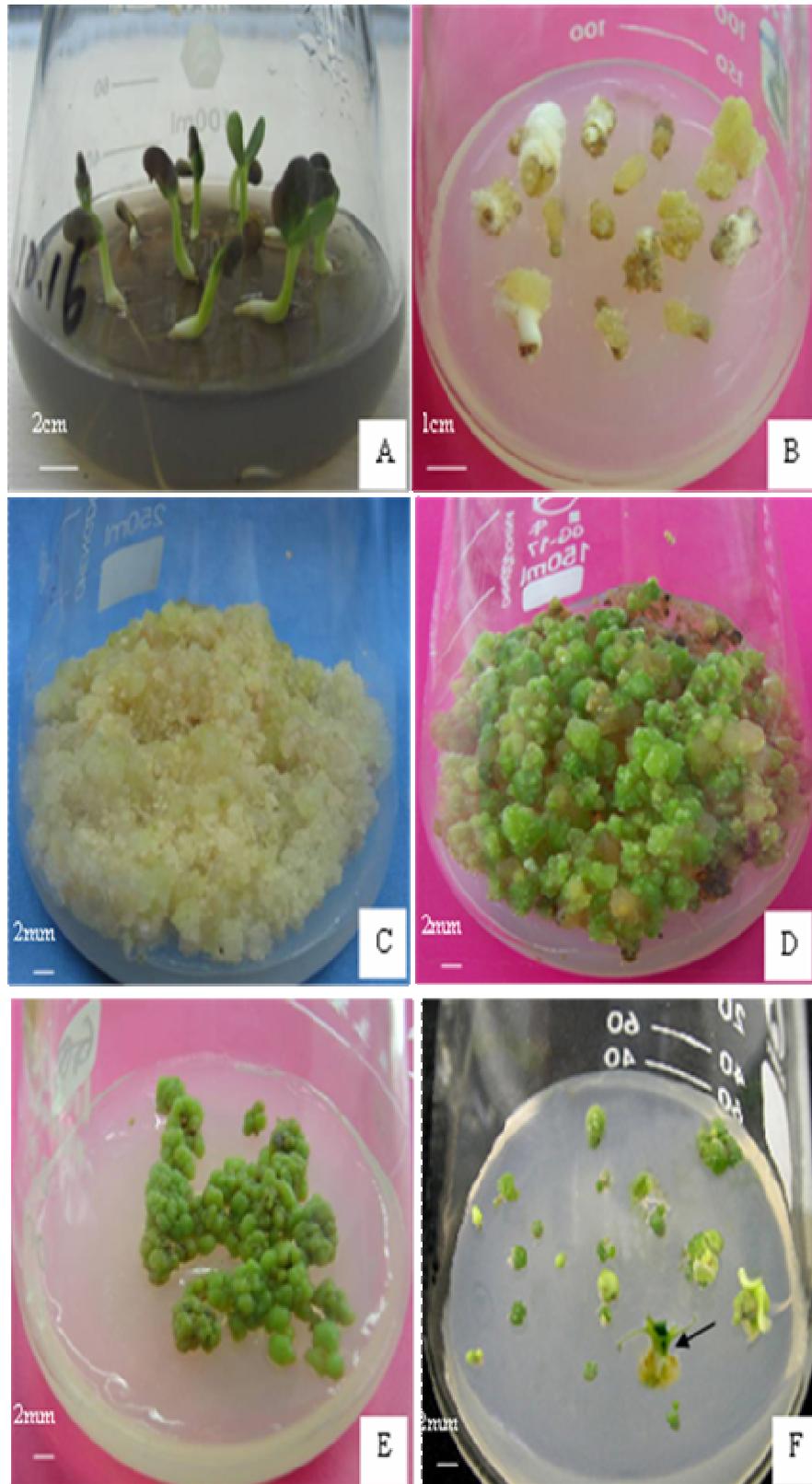


Figure 1. Embryogenesis of *G. glabra*. A = Aseptic explant preparation, bar = 2cm; B = callus induction from hypocotyl of *G. glabra*, bar = 1 cm; C = subculture of calli; D = embryo formation; E = well-developed embryos on medium with 1000 mg/L ME; F = shoot induction (see arrow); C – F, bar = 2 mm.

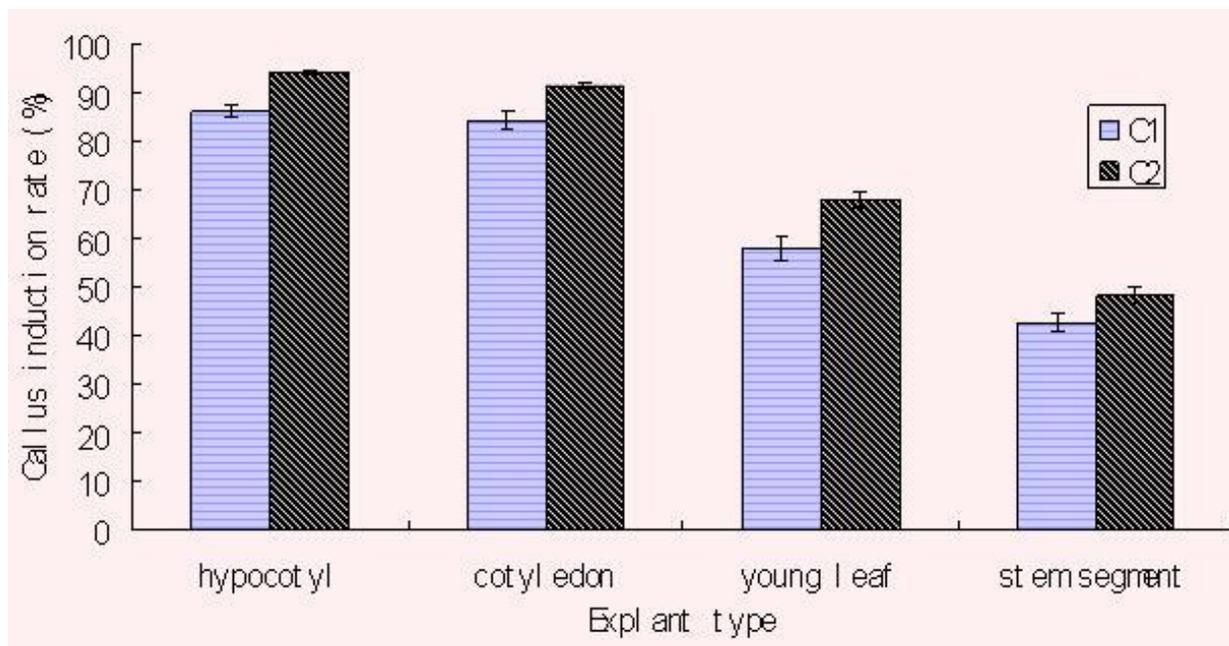


Figure 2. The influences of different explants of *G. glabra* on callus formation rate.

(0.5 mg/L) + IBA (0.1 mg/L) medium. To promote the development of somatic embryos, several chemicals, such as lactalbumin hydrolysate (LH), dasein hydrolysate (DH), ME, glycerol and active carbon were supplemented in the medium. The results showed that the medium with 500 mg/L ME was favorable for embryo development (data not show). So, more works on the effects of ME concentration on the embryo development were carried out. The results showed that the embryos became brown or died when cultured on the medium supplemented with 1500 mg/L ME; further embryo development can be seen on the medium supplemented with ME of both 500 and 1000 mg/L. The embryo multiplication index were 8.24 ± 0.50 on medium with 500 mg/L ME and the diameter of the embryo were 1.0-2.0 mm, averagely. The multiplication index of the embryo on medium with 1000 mg/L ME were 9.34 ± 0.65 and the diameter were 2.0 - 5.0 mm, averagely (Figure 1E), but only a few weak shoots were obtained (Figure 1F) and most of these embryos were reluctant to be redifferentiated to shoots when transfer to shoot induction medium.

Histological observation of the somatic embryogenesis

In our research, two kinds of calli, embryogenic and non-embryogenic, were obtained. The former type could grow well and many green globular embryos were observed in the surface of the calli on embryo induction medium, which developed further on the embryo maturing medium. By contrast, the latter type calli turned brown or

died when they were transferred to embryo induction medium although they grew well on the calli induction medium.

To explore the origin of the embryo, the embryogenic calli, non-embryogenic calli and globular somatic embryo were subjected to histological observation. Notable difference was detected on cell morphology between the embryogenic and non-embryogenic calli. In the non-embryogenic calli, a number of oblong-shaped cells with inconspicuous nuclei were observed (Figure 3A), while the embryogenic calli contained round and oval cells with conspicuous nuclei (Figure 3B). Microscopic observation (Figure 3C) and histological section (Figures 3D - F) showed that the green globular somatic embryo (Figure 1E) were composed of many small green globular embryo submerged in translucent calli.

In our research, we found that the callus and embryo induction of the *G. glabra* were easy, but the re-differentiation of the embryo are relatively difficult, in agreement with the *G. uralensis* Fisch (Rei et al., 1986). When the induced embryos were transferred to the medium added with 1000 mg/L ME, they could develop further. Unfortunately, only a few weak shoots were obtained and most of them were recalcitrant to re-differentiation on the differentiation medium.

Microscopic observation and histological section showed that the green globular somatic embryos were actually made up by many small green globular embryos surrounded by translucent calli and most of them were just under the initial differentiation stage. This gives a hint that the low embryoid regeneration in our research may be due to the fact that the embryoids were not well matured.

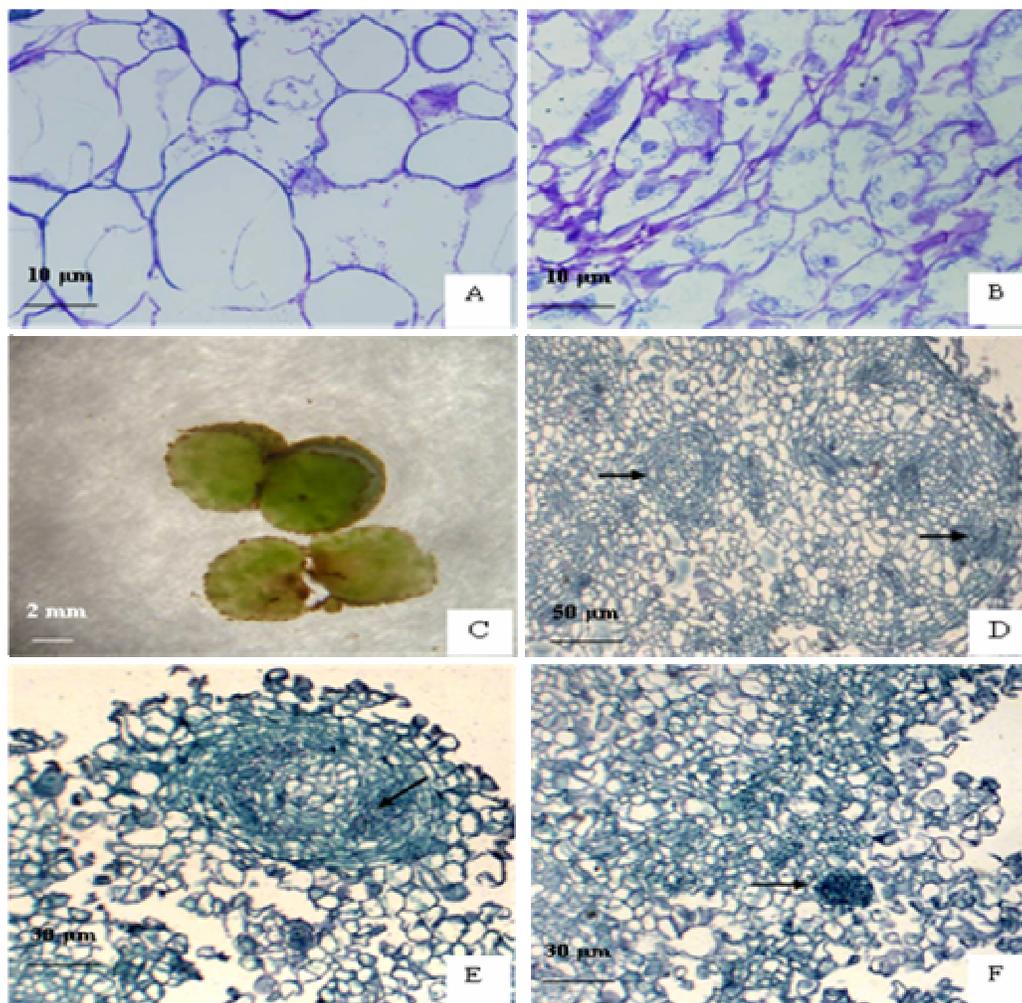


Figure 3. Characteristics of the embryo formation of *G. glabra* by microscopic and histological observation. A = Cell characteristic of the non-embryogenic calli, bar = 10 µm; B = cell characteristic of the embryogenic calli, bar = 10 µm; C = cut surface morphology of the embryo, bar = 2 mm. D, E = histological observation of the embryo-like structure. Arrows show the meristem region in the embryo-calli; D, bar = 50 µm; E, bar = 30 µm; F = the global embryooid formed (arrow), bar = 30 µm.

ACKNOWLEDGMENTS

Our sincere appreciation goes to Prof. Liu Jihong and Dr. Chen Chunli from Huazhong Agricultural University for critical reading of the manuscript and technical support. The study was partially funded by New Century Excellent Talents Program of Ministry of Education of China (NCET-06-0646) and National “11th Five-Year Plan” to Support Science and Technology Project of China (2008BAI63B04).

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